

# Evidence for non-cysteinyl coordination of the [2Fe-2S] cluster in *Escherichia coli* succinate dehydrogenase

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The consequences of replacing Cys<sup>65</sup> in the FrdB subunit of *Escherichia coli* fumarate reductase by Asp or Ala have been investigated in terms of bacterial growth, enzymatic activity, and the EPR/redox properties of the [2Fe-2S] cluster. An aspartic acid residue occupies the equivalent position in *E. coli* succinate dehydrogenase, and the FrdBCys<sup>65</sup>Asp mutation has little effect on cell growth, enzyme activity or the physical properties of the Frd [2Fe-2S] cluster. In contrast, the [2Fe-2S] cluster was not observed in the FrdBCys<sup>65</sup>Ala mutant showing that a coordinating residue is required at this position for assembly of this cluster and significant levels of enzymatic activity. These results support the presence of one non-cysteinyl, oxygenic ligand for the [2Fe-2S] cluster in *E. coli* succinate dehydrogenase.

Site-directed mutagenesis; [2Fe-2S] cluster; EPR; Fumarate reductase; Succinate dehydrogenase

## 1. INTRODUCTION

Based on the available EPR and amino acid sequence data it is probable that all fumarate reductases and succinate dehydrogenases, irrespective of their animal or bacterial origin, contain three distinct types of Fe-S cluster: Center 1, a [2Fe-2S]<sup>2+</sup> cluster; Center 2, a [4Fe-4S]<sup>2+</sup> cluster; Center 3, a [3Fe-4S]<sup>+</sup> cluster [1,2]. Recent electrochemical and spectroscopic studies of mutant forms of the fumarate reductase from *Escherichia coli* in which individual cysteine residues were mutated to serines, provided the first definitive evidence that the four cysteinyl residues located near the N-terminus of FrdB subunit are involved in the ligation of Center 1 [3]. The arrangement and location of these four cysteine residues are conserved in all iron-sulfur protein (Ip) subunits of fumarate reductases and succinate dehydrogenases for which the sequences are known [3], with the notable exception of the succinate dehydrogenase from *E. coli* [4], which has an aspartic acid residue in place of the third cysteine (Cys<sup>65</sup> in *E. coli* fumarate reductase). However, this apparent difference in the ligation of Center 1 in *E. coli* succinate dehydrogenase is not reflected in anomalous EPR or redox

properties for this cluster [5,6]. To assess further the importance of the nature of the residue at this position on the assembly and properties of Center 1 and the enzymatic activity, we have constructed and characterized *E. coli* fumarate reductase mutants in which Cys<sup>65</sup> of Ip is replaced by Asp (FrdBCys<sup>65</sup>Asp mutant) or Ala (FrdBCys<sup>65</sup>Ala mutant). The results are compared with those obtained for the wild-type and FrdBCys<sup>65</sup>Ser mutant [3].

## 2. MATERIALS AND METHODS

The *E. coli* strains used for growth and biochemical measurements have been described previously [7]. DW12 contains a deletion of the *frd* operon (*ΔfrdABCD*) and strain DW35 contains the *frd* deletion as well as a disruption of the *sdh* operon (*sdhC::kan*). Both strains are devoid of fumarate reductase activity, and are incapable of growth on anaerobic glycerol/fumarate medium, unless they contain a plasmid encoding a functional fumarate reductase complex. Site-directed mutagenesis of *frdB* was performed as in previous work [3] with oligonucleotides designed to change Cys<sup>65</sup> of FrdB to either Ser, Asp or Ala. The mutations were confirmed by DNA sequence analysis of both the single-stranded phage used for construction and the double-stranded DNA of the plasmid used for expression of the mutant protein [3]. Growth studies, the preparation of cytoplasmic membrane fractions, and the catalytic assays were carried out as previously described [3,7–9].

EPR samples of cytoplasmic membrane preparations of *E. coli* DW12 or DW35 with plasmid-amplified expression of wild-type or mutant fumarate reductase were oxidized with 20 mM fumarate or reduced with 20 mM succinate or 10 mM dithionite. Dye-mediated EPR redox titrations were carried out as previously described [3]. Reduction potentials were measured at pH 7.0 with a platinum working electrode and a Ag/AgCl reference electrode and are reported

Abbreviations: BV<sub>red</sub>, reduced benzyl viologen; MQH<sub>2</sub>, menaquinol-6; PMS, phenazine methosulfate.

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relative to the normal hydrogen electrode. EPR spectra were recorded at X-band using an IBM/Bruker ER200D spectrometer with data storage, and manipulation performed on an ESP 1600 data processing system. Sample temperatures in the range 4–80 K were obtained using an Oxford Instruments ESR-9 cryostat.

### 3. RESULTS

Growth data for *E. coli* DW35 with plasmid-amplified expression of wild-type, as well as FrdBCys<sup>65</sup>Ser, FrdBCys<sup>65</sup>Asp, and FrdBCys<sup>65</sup>Ala mutant fumarate reductases, plus the enzymatic activities for cytoplasmic membrane preparations are shown in Table I. Compared to the wild-type control, the FrdBCys<sup>65</sup>Ser and FrdCys<sup>65</sup>Asp mutations had no significant effect on the rate of cell growth under conditions requiring a functional fumarate reductase (i.e. anaerobic growth on glycerol/fumarate medium or aerobic growth on minimal succinate medium). In contrast, no growth was observed for the FrdBCys<sup>65</sup>Ala mutant under either conditions. With non-physiological electron donors and acceptors, the FrdBCys<sup>65</sup>Ser and FrdBCys<sup>65</sup>Asp mutants both had slightly impaired fumarate reductase and succinate dehydrogenase activities compared to wild-type, i.e. ~15% decrease in fumarate:BV<sub>red</sub> oxidoreductase activity and ~50% decrease in succinate:PMS oxidoreductase activity. However, fumarate reductase activity using the physiological electron donor, MQH<sub>2</sub>, was comparable to wild-type for the FrdBCys<sup>65</sup>Asp mutant, and significantly increased for the FrdBCys<sup>65</sup>Ser mutant. In accord with the growth data, the FrdBCys<sup>65</sup>Ala mutation resulted in drastically reduced enzymatic activity in the MQH<sub>2</sub>-fumarate reductase assay and both of the non-physiological enzyme assays. The growth and activity data reported here for the FrdBCys<sup>65</sup>Ser mutant relative to wild-type are in good agreement with previous data obtained with *E. coli* DW12 (*ΔfrdABCD*) [3].

EPR spectra of fumarate-oxidized and dithionite-reduced cytoplasmic membrane samples of *E. coli* DW35 with plasmid-amplified wild-type, FrdBCys<sup>65</sup>Ser,

FrdBCys<sup>65</sup>Asp and FrdBCys<sup>65</sup>Ala fumarate reductases are shown in Fig. 1. Identical spectra were observed for samples prepared using either the DW12 or DW35 *E. coli* strains. The presence of Center 3 in all three mutants is shown by the observation of the characteristic EPR resonance of the  $S = 1/2$  [3Fe-4S]<sup>+</sup> cluster,  $g = 2.016, \sim 1.98, \sim 1.93$ , in the fumarate-oxidized membranes [10]. As predicted from the activity data, fumarate was not effective in oxidizing the FrdBCys<sup>65</sup>Ala mutant and identical EPR spectra were observed for the membranes as prepared in the absence of fumarate. EPR signals indicative of a  $S = 1/2$  [2Fe-2S]<sup>+</sup> cluster, Center 1, i.e.  $g_z = 2.01-2.03, g_y = 1.92-1.93, g_x = 1.92-1.93$  and observable without significant broadening at 70 K, are observed in the succinate- and dithionite-reduced samples of the FrdBCys<sup>65</sup>Ser and FrdBCys<sup>65</sup>Asp mutants, but not the FrdBCys<sup>65</sup>Ala mutant. The presence of the dithionite-reducible  $S = 1/2$  [4Fe-4S]<sup>2+,+</sup> cluster, Center 2, in *E. coli* fumarate reductase can be assessed by dramatic enhancement of the spin relaxation of Center 1 in dithionite- vs. succinate-reduced samples, or by direct observation of a broad fast-relaxing resonance, with features to high- and low-field of the Center 1 resonance, at temperatures below 20 K in dithionite-reduced samples [11,12]. Based on both criteria, Center 2, is present in both the FrdBCys<sup>65</sup>Ser and FrdBCys<sup>65</sup>Asp mutants. The enhancement in spin relaxation is shown by the more than 100-fold increase in the Center 1 half-saturation parameter,  $P_{1/2}$ , that accompanies dithionite reduction (see Table II). The dithionite reduced samples of these mutants also exhibited a broad resonance at 10 K with features at  $g = 2.17, 2.06, 1.82$  and  $1.65$  (data not shown), identical to that observed and attributed to reduced Center 2 in the wild-type membranes. However, no additional  $S = 1/2$  resonances, over and above those observed for the control samples of the fumarate reductase deletion strains, DW12 and DW35, were observed in the dithionite-reduced samples of the FrdBCys<sup>65</sup>Ala mutant. Although this suggests that Center 2, in addition to Center 1, is not assembled in the FrdBCys<sup>65</sup>Ala

Table I  
Growth properties and enzyme activities for *E. coli* DW35 with plasmid-amplified expression of wild-type and mutant fumarate reductases

Plasmid	Doubling time (h)		Turnover number <sup>a</sup> (min <sup>-1</sup> )		
	-O <sub>2</sub> <sup>b</sup>	+O <sub>2</sub> <sup>c</sup>	BV <sub>red</sub> -fumarate oxidoreductase	MQH <sub>2</sub> -fumarate oxidoreductase	Succinate-PMS oxidoreductase
pH3 wild-type	2.2	2.1	30,200	18,700	10,400
pH3 FrdBCys <sup>65</sup> Ser	2.5	2.9	26,500	27,300	5,600
pH3 FrdBCys <sup>65</sup> Asp	2.6	2.4	25,000	17,600	4,500
pH3 FrdBCys <sup>65</sup> Ala	n.g.	n.g.	900	400	300

<sup>a</sup> Turnover numbers based on 1 mol of histidyl-FAD per mol of enzyme for cytoplasmic membrane fractions.

<sup>b</sup> Anaerobic growth on glycerol/fumarate medium.

<sup>c</sup> Aerobic growth on succinate minimal media.  
n.g., no growth.

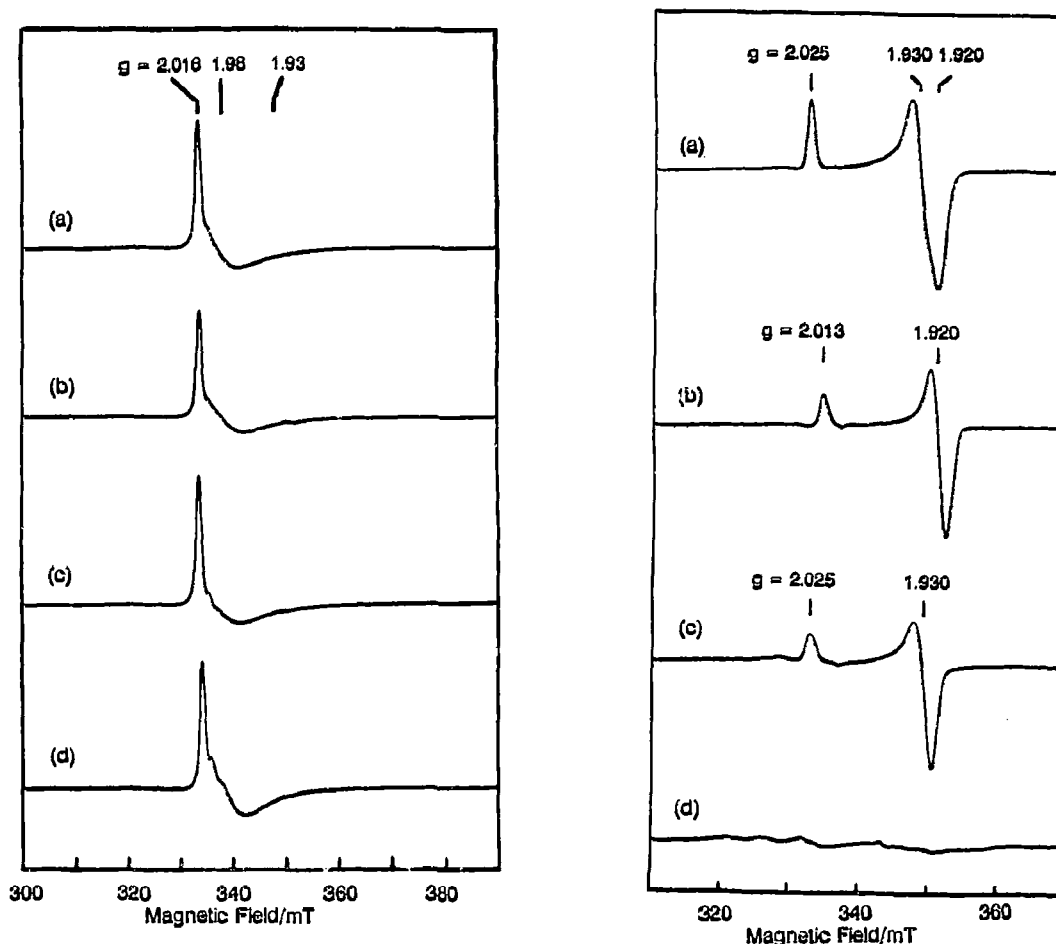


Fig. 1. X-band EPR spectra of fumarate-oxidized (left panel) and dithionite-reduced (right panel) cytoplasmic membrane preparations of *E. coli* DW35 with plasmid-amplified expression of wild-type and mutant forms of fumarate reductase. (a) Wild-type. (b) FrdBCys<sup>65</sup>Ser mutant. (c) FrdBCys<sup>65</sup>Asp mutant. (d) FrdBCys<sup>65</sup>Ala mutant. The relative spectrometer gains are the same for each pair of fumarate-oxidized and dithionite-reduced samples. Conditions: temperature, 13 K; microwave power, 1 mW; modulation amplitude, 1 mT; frequency, 9.44 GHz.

mutant, we cannot rule out the possibility that it is assembled as a [4Fe-4S] cluster that is either no longer reducible by dithionite or is reduced but has a  $S = 3/2$  ground state. Broad EPR signals from  $S = 3/2$  [4Fe-4S]<sup>+</sup>

Table II

EPR properties and midpoint potentials of Center I in cytoplasmic membranes of *E. coli* DW12 with plasmid-amplified wild-type and mutant fumarate reductases

Plasmid	EPR $g$ values	$P_{1/2}$ values <sup>a</sup> (mW)		
		Succinate	Dithionite	$E_m^b$ (mV)
pH3 wild-type	2.025, 1.930, 1.920	0.30	100	-79 <sup>c</sup>
pH3 FrdBCys <sup>65</sup> Ser	2.013, 1.920, 1.920	0.45	70	-49 <sup>c</sup>
pH3 FrdBCys <sup>65</sup> Asp	2.025, 1.930, 1.930	0.25	80	-76
pH3 FrdBCys <sup>65</sup> Ala	n.o.	—	—	—

<sup>a</sup> Half-saturation powers at 10 K for succinate- and dithionite-reduced samples.

<sup>b</sup> pH 7.0 vs. standard hydrogen electrode.

<sup>c</sup> Taken from [3].

n.o., not observed.

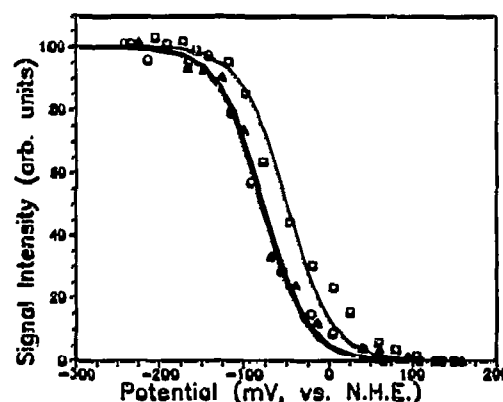


Fig. 2. EPR redox titrations for Center I in cytoplasmic membrane preparations of *E. coli* DW12 with plasmid-amplified expression of wild-type and mutant forms of fumarate reductase. The peak-to-trough EPR intensity at 35 K and 1 mW microwave power of the derivative-shaped feature, centered between  $g = 1.93$  and  $1.92$ , is plotted as a function of the potential measured relative to the normal hydrogen electrode. The solid lines represent least-squares fits of the data to a one-electron Nernst equation. (○) Wild-type,  $E_m = -79$  mV; (□), FrdBCys<sup>65</sup>Ser mutant,  $E_m = -49$  mV; (△) FrdBCys<sup>65</sup>Asp mutant,  $E_m = -76$  mV.

clusters would be extremely difficult to detect in membrane samples. Further spectroscopic studies of the isolated tetrameric complex are planned to evaluate these possibilities.

As previously reported [3], the effect of the FrdBCys<sup>65</sup>Ser mutation on Center 1 is manifest by a small change in EPR  $g$  values and a 30 mV increase in mid-point potential compared to wild-type. In contrast, the FrdBCys<sup>65</sup>Asp mutation results in no significant change in mid-point potential of Center 1 and EPR  $g$  values that are almost identical to wild-type except for slightly less pronounced rhombic splitting on  $g_{\perp}$  (see Table II and Figs. 1 and 2). We conclude that neither the fumarate reductase activity nor the properties of Center 1 are significantly perturbed by the FrdBCys<sup>65</sup>Asp mutation in *E. coli* fumarate reductase.

#### 4. DISCUSSION

With the notable exception of the Rieske protein, X-ray crystallographic studies of electron transfer proteins containing [2Fe-2S], [3Fe-4S] and [4Fe-4S] centers have shown these clusters to be coordinated exclusively by cysteinyl S. However, in some bacterial ferredoxins, notably *Desulfovibrio africanus* FdIII [13] and *Pyrococcus furiosus* Fd [14], an aspartate residue is found in place of one of the conserved cysteine residues resulting in [4Fe-4S]<sup>2+,•</sup> clusters with one non-cysteinyl ligand. The results presented here serve to demonstrate that one of the cysteines that is involved in ligating the [2Fe-2S] cluster in *E. coli* fumarate reductase can be replaced by aspartate without significant perturbation of the spectroscopic, catalytic or redox properties. Moreover, it is clear that Center 1 in *E. coli* succinate dehydrogenase provides an in vivo example of a [2Fe-2S] cluster with the same type of anomalous coordination, i.e. one non-cysteinyl ligand.

The absence of Center 1 in the FrdBCys<sup>65</sup>Ala mutant shows that a residue with potential for metal coordination such as Cys, Ser or Asp is required at position 65 for assembly of the [2Fe-2S] cluster in *E. coli* FrdB. Whether these residues coordinate directly to Fe or indirectly by hydrogen bonding to a coordinated water molecule remains to be established. The relatively minor changes in  $g$  value anisotropy and mid-point potential that accompany mutation of Cys<sup>65</sup> for Ser or Asp can be rationalized in terms of changes in coordination provided it is occurring at the localized valence Fe(III) site of the cluster. More dramatic changes would only be expected, and are observed, for mutations that perturb the ligand field on the localized valence Fe(II) site [3].

We have considered the possibility whereby aspartate coordination to Center 1 may be one of several factors that determine whether the enzyme functions as a fumarate reductase or succinate dehydrogenase. This is

unlikely for several reasons. First, all other succinate dehydrogenases that have been sequenced thus far have a conserved cysteine in the position analogous to Cys<sup>65</sup> in *E. coli* FrdB. Second, the FrdBCys<sup>65</sup>Asp mutant retained its native fumarate reductase activity while undergoing a 50% decrease in succinate dehydrogenase activity with PMS as the electron acceptor. Third, since the redox potential of Center 1 is unchanged in the FrdBCys<sup>65</sup>Asp mutant, it is unlikely that aspartate coordination alone can be invoked to explain the increase in the mid-point potential of this cluster in *E. coli* succinate dehydrogenase compared to *E. coli* fumarate reductase. In general, Center 1 has a lower potential in fumarate reductases ( $E_m = -20$  mV to  $-80$  mV) compared to succinate dehydrogenases ( $E_m = -10$  mV to  $+80$  mV), which is consistent with the opposite direction of electron flow [2]. However, the activity results for the FrdBCys<sup>65</sup>Ser mutant, in which the mid-point potential of Center 1 is increased by 30 mV, demonstrate that increasing the potential of Center 1 does not necessarily equate to enhanced succinate dehydrogenase activity.

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